PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 9/10, 15/11, 15/54 15/82, C12P 19/04

A3

(11) International Publication Number: **WO 97/33974**

(43) International Publication Date: 18 September 1997 (18.09.97)

(21) International Application Number: PCT/EP97/01117

(22) International Filing Date: 7 March 1997 (07.03.97)

(30) Priority Data:
9605274.1 13 March 1996 (13.03.96) GB

(71) Applicant *(for all designated States except US)*: DANISCO A/S [DK/DK]; Langebrogade 1, P.O. Box 17, DK-1001 Copenhagen K (DK).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: DAL DEGAN, Florence [FR/DK]; (DK). POULSEN, Peter [DK/DK]; (DK). MARCUSSEN, Jan [DK/DK]; (DK). OXENBAL, Susanne [DK/DK]; Danisco a/s, Langebrogade 1, P.O. Box 17, DK-1001 Copenhagen K (DK).

(74) Agents: HARDING, Charles, Thomas et al.; D. Young & Co., 21 New Fetter Lane, London EC4A 1DA (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
30 October 1997 (30.10.97)

(54) Title: DNA ENCODING ALPHA-1(1,4)-GLUCAN ACETYL-TRANSFERASE

(57) Abstract

An enzyme is described. The enzyme has α(1,4) glucan acetyl-transferase activity.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N9/10 C12N15/11 C12N15/54 C12N15/82 C12P19/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE 44 25 688 A (A U F ANALYTIK UMWELTTECHNIK F) 18 January 1996 see the whole document ---	14
X	BRAND B. AND BOOS W.: "Maltose transacetylase of Escherichia coli" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 266, no. 21, 25 July 1991, pages 14113-14118, XP000676505 cited in the application see the whole document ---	1-15
P,X	ROBERTS D. ET AL.: "Sequence of minutes 4-25 of E. coli, AC U82664" EMBL DATABASE, 19 January 1997, HEIDELBERG, XP002036098 see the whole document -----	1-12,15

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 September 1997	23. 09. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Kania, T

Form PCT/ISA/210 (second sheet) (July 1992)

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE 4425688 A	18-01-96	NONE	

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N

A2

(11) International Publication Number: **WO 97/33974**

(43) International Publication Date: 18 September 1997 (18.09.97)

(21) International Application Number: PCT/EP97/01117

(22) International Filing Date: 7 March 1997 (07.03.97)

(30) Priority Data:
9605274.1 13 March 1996 (13.03.96) GB

(71) Applicant *(for all designated States except US)*: DANISCO A/S [DK/DK]; Langebrogade 1, P.O. Box 17, DK-1001 Copenhagen K (DK).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: DAL DEGAN, Florence [FR/DK]; (DK). POULSEN, Peter [DK/DK]; (DK). MARCUSSEN, Jan [DK/DK]; (DK). OXENBAL, Susanne [DK/DK]; Danisco a/s, Langebrogade 1, P.O. Box 17, DK-1001 Copenhagen K (DK).

(74) Agents: HARDING, Charles, Thomas et al.; D. Young & Co., 21 New Fetter Lane, London EC4A 1DA (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: AN ENZYME

(57) Abstract

An enzyme is described. The enzyme has $\alpha(1,4)$ glucan acetyl-transferase activity.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia
AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CS	Czechoslovakia
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgystan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakhstan
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam

AN ENZYME

The present invention relates to an enzyme. The present invention also relates to a nucleotide sequence coding for the enzyme.

Boos and coworkers in 1981 and 1982 (1, 2) presented evidence for the existence of an enzyme capable of acetylating maltose *via* transfer of the acetyl group from Acetyl-coenzyme A to maltose in *E. coli*. In particular, Boos *et al* (1) observed the formation of acetyl-maltose and acetyl-oligomaltosides after accumulation of maltose or maltooligosides in *E. coli*. They also observed the formation of acetyl-maltose and acetyl-oligomaltosides *in vitro* when maltose or maltotriose, acetyl-coenzyme A and a cytosolic *E. coli* extract were mixed together Boos *et al* (2).

Boos *et al* in 1981 stated that the activity responsible for maltose and maltodextrin acetylation was unknown. However, in their further studies of 1982 (2), Feundlieb and Boos named the unknown enzyme "maltose transacetylase" but then said that the function of maltose transacetylase in *E. coli* was unclear.

Later Brand and Boos (3) isolated an *E. coli* mutant lacking the gene encoding maltose transacetylase. This mutant enabled them to map the gene at 10.4 min on the *E. coli* linkage map. In addition, they cloned a 3.4 kb DNA fragment containing the gene in a high copy plasmid. Over-expressed maltose transacetylase was then purified to homogeneity from cell free extracts of an *E. coli* strain harbouring the above mentioned plasmid. The enzyme was shown to be a homodimer with two identical subunits of 20 kDa. The km (mM) and Vmax (μmol/min x mg enzyme) values of this enzyme for the substrates glucose, maltose and acetyl-coenzyme A were 62 and 200, 90 and 110, and 0.018 and 166 respectively. Maltotriose and other oligosaccharides were found to be acetylated with a rate of 2% of the rate determined for glucose. In addition, Brand and Boos presented the following relative transacetylation rates: glucose 1, maltose 0.55, mannose 0.2, fructose 0.07, galactose 0.04, maltotriose and other malto-oligosaccharides 0.02. Oligosaccharides are saccharides having less than ten sugar units.

Despite of these findings Brand and Boos did not sequence the enzyme or the nucleotide sequence coding for the maltose transacetylase enzyme.

According to a first aspect of the present invention there is provided an enzyme having $\alpha(1,4)$ glucan acetyl-transferase activity, wherein the enzyme comprises the amino acid sequence shown as SEQ ID No. 1, or a variant, homologue or fragment thereof.

According to a second aspect of the present invention there is provided a recombinant enzyme having $\alpha(1,4)$ glucan acetyl-transferase activity, wherein the enzyme comprises the amino acid sequence shown as SEQ ID No. 1, or a variant, homologue or fragment thereof.

According to a third aspect of the present invention there is provided a recombinant enzyme having $\alpha(1,4)$ glucan acetyl-transferase activity, wherein the enzyme has the amino acid sequence shown as SEQ ID No. 1.

According to a fourth aspect of the present invention there is provided a recombinant enzyme having $\alpha(1,4)$ glucan acetyl-transferase activity, wherein the recombinant enzyme is immunologically reactive with an antibody raised against a purified recombinant enzyme according to the above-mentioned aspect of the present invention.

According to a fifth aspect of the present invention there is provided a nucleotide sequence coding for the enzyme of the present invention or a sequence that is complementary thereto.

According to a sixth aspect of the present invention there is provided a nucleotide sequence comprising the sequence shown as SEQ ID No. 2, or a variant, homologue or fragment thereof or a sequence that is complementary thereto.

According to a seventh aspect of the present invention there is provided a nucleotide sequence having the sequence shown as SEQ ID No. 2.

According to an eighth aspect of the present invention there is provided a construct comprising or expressing the nucleotide sequence or the enzyme of the present invention.

According to a ninth aspect of the present invention there is provided a vector comprising or expressing the construct or the nucleotide sequence or the enzyme according to the present invention.

According to a tenth aspect of the present invention there is provided a plasmid comprising or expressing the vector, the construct or the nucleotide sequence or the enzyme according to the present invention.

According to an eleventh aspect of the present invention there is provided a transgenic organism comprising or expressing the plasmid, the vector, the construct or the nucleotide sequence or enzyme according to the present invention.

According to a twelfth aspect of the present invention there is provided a modified carbohydrate (preferably starch) prepared by a method comprising or expressing or using the present invention.

The enzyme of the present invention may be obtainable from any one of a bacterium, a fungus, an alga, a yeast, or a plant. Preferably, the enzyme is obtainable from *E.coli*.

The $\alpha(1,4)$ glucan acetyl-transferase of the present invention is sometimes referred to as Mac. The gene coding for the $\alpha(1,4)$ glucan acetyl-transferase of the present invention is also sometimes referred to as the *mac* gene.

According to a seventh aspect of the present invention there is provided a nucleotide sequence having the sequence shown as SEQ ID No. 2.

According to an eighth aspect of the present invention there is provided a construct comprising or expressing the nucleotide sequence or the enzyme of the present invention.

According to a ninth aspect of the present invention there is provided a vector comprising or expressing the construct or the nucleotide sequence or the enzyme according to the present invention.

According to a tenth aspect of the present invention there is provided a plasmid comprising or expressing the vector, the construct or the nucleotide sequence or the enzyme according to the present invention.

According to an eleventh aspect of the present invention there is provided a transgenic organism comprising or expressing the plasmid, the vector, the construct or the nucleotide sequence or enzyme according to the present invention.

According to a twelfth aspect of the present invention there is provided a modified carbohydrate (preferably starch) prepared by a method comprising or expressing or using the present invention.

The enzyme of the present invention may be obtainable from any one of a bacterium, a fungus, an alga, a yeast, or a plant. Preferably, the enzyme is obtainable from *E.coli*.

The $\alpha(1,4)$ glucan acetyl-transferase of the present invention is sometimes referred to as Mac. The gene coding for the $\alpha(1,4)$ glucan acetyl-transferase of the present invention is also sometimes referred to as the *mac* gene.

Preferably, the enzyme comprises the amino acid sequence shown as SEQ ID No 1, or a variant, homologue or fragment thereof.

Preferably, the enzyme has the amino acid sequence shown as SEQ ID No 1.

Preferably, the enzyme is encoded by a nucleotide sequence comprising the nucleotide sequence shown as SEQ ID No 2, or a variant, homologue or fragment thereof or a sequence that is complementary thereto.

Preferably, the enzyme is encoded by the nucleotide sequence shown as SEQ ID No 2.

Preferably, the organism is a plant.

Preferably, the nucleotide sequence is a DNA sequence.

The enzyme or nucleotide sequence(s) coding for same may be used *in vitro* or *in vivo* in combination with one or more other enzymes or nucleotide sequence(s) coding for same, which enzymes or nucleotide sequence(s) coding for same are preferably prepared by use of recombinant DNA techniques.

Thus, according to one aspect of the present invention, an *in vivo* enzymatic modification process can be followed by an *in vitro* enzymatic modification process. In these modification steps, the enzymes used need not necessarily be the same enzymes.

The terms "variant", "homologue" or "fragment" in relation to the enzyme include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) amino acid from or to the sequence providing the resultant amino acid sequence has $\alpha(1,4)$ glucan acetyl-transferase activity, preferably having at least the same activity of the enzyme shown as SEQ ID No. 1. In particular, the term "homologue" covers homology with respect to structure and/or function

providing the resultant enzyme has α(1,4) glucan acetyl-transferase activity. With respect to sequence homology, preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology to the sequence shown as SEQ ID No. 1. More preferably there is at least 95%, more preferably at least 98%, homology to the sequence shown as SEQ ID No. 1.

The terms "variant", "homologue" or "fragment" in relation to the nucleotide sequence coding for the enzyme include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acid from or to the sequence providing the resultant nucleotide sequence codes for an enzyme having α(1,4) glucan acetyl-transferase activity, preferably having at least the same activity of the enzyme shown as SEQ ID No. 1. In particular, the term "homologue" covers homology with respect to structure and/or function providing the resultant nucleotide sequence codes for an enzyme having α(1,4) glucan acetyl-transferase activity. With respect to sequence homology, preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology to the sequence shown as SEQ ID No. 2. More preferably there is at least 95%, more preferably at least 98%, homology to the sequence shown as SEQ ID No. 2.

The above terms are synonymous with allelic variations of the sequences.

The term "complementary" means that the present invention also covers nucleotide sequences that can hybridise to the nucleotide sequence of the present invention.

The term "nucleotide" in relation to the present invention includes genomic DNA, cDNA, synthetic DNA, and RNA. Preferably it means DNA, more preferably cDNA for the coding sequence of the present invention.

Preferably the nucleotide sequence is not a native nucleotide sequence. In this regard, the term "native nucleotide sequence" means an entire nucleotide sequence that is in its native environment and when operatively linked to an entire promoter with which it is naturally associated, which promoter is also in its native environment.

Thus, the enzyme of the present invention can be expressed by a nucleotide sequence in its native organism but wherein the nucleotide sequence is not under the control of the promoter with which it is naturally associated within that organism.

The enzyme of the present invention may be used in conjunction with other enzymes.

Preferably the enzyme is not a native enzyme. In this regard, the term "native enzyme" means an entire enzyme that is in its native environment and when it has been expressed by its native nucleotide sequence.

The term "construct" - which is synonymous with terms such as "conjugate", "cassette" and "hybrid" - includes the nucleotide sequence directly or indirectly attached or fused to a promoter. An example of an indirect attachment is the provision of a suitable spacer group such as an intron sequence, such as the *Sh1*-intron or the ADH intron, intermediate the promoter and the nucleotide sequence.

In each case, it is highly preferred that the terms do not cover the natural combination of the gene coding for the enzyme ordinarily associated with the wild type gene promoter and when they are both in their natural environment. One highly preferred embodiment of the present invention therefore relates to the nucleotide sequence of the present invention operatively linked to a heterologous promoter.

The construct may even contain or express a marker which allows for the selection of the genetic construct in, for example, a plant, such as potato, into which it has been transferred. Various markers exist which may be used, such as for example those encoding mannose-6-phosphate isomerase (especially for plants) or those markers that provide for antibiotic resistance - e.g. resistance to G418, hygromycin, bleomycin, kanamycin and gentamycin.

The term "vector" includes expression vectors and transformation vectors.

The term "expression vector" means a construct capable of *in vivo* or *in vitro*

expression.

The term "transformation vector" means a construct capable of being transferred from one species to another - such as from an *E.coli* plasmid to an *Agrobacterium* to a plant.

The term "tissue" includes tissue and organ, which tissue and organ can be isolated tissue and isolated organ, as well as tissue and organ when within an organism.

The term "organism" in relation to the present invention includes any organism that could comprise the nucleotide sequence coding for the enzyme according to the present invention and/or products obtained therefrom, and/or wherein the nucleotide sequence according to the present invention can be expressed when present in the organism.

Preferably the organism is a plant.

The term "transgenic organism" in relation to the present invention includes any organism that comprises the nucleotide sequence coding for the enzyme according to the present invention and/or the products obtained therefrom, and/or wherein the nucleotide sequence according to the present invention can be expressed within the organism. Preferably the nucleotide sequence is incorporated in the genome of the organism.

Preferably the transgenic organism is a plant.

Therefore, the transgenic organism of the present invention includes an organism comprising any one of, or combinations of, the nucleotide sequence coding for the enzyme according to the present invention, constructs according to the present invention, vectors according to the present invention, plasmids according to the present invention, cells according to the present invention, tissues according to the present invention, or the products thereof. For example the transgenic organism can

also comprise the nucleotide sequence coding for the enzyme of the present invention under the control of a heterologous promoter. The transgenic organism does not comprise the combination of a promoter and the nucleotide sequence coding for the enzyme according to the present invention, wherein both the promoter and the nucleotide sequence are native to that organism and are in their natural environment.

The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site in the Jacob-Mond theory of gene expression.

The promoter could additionally include one or more features to ensure or to increase expression in a suitable host. For example, the features can be conserved regions such as a Pribnow Box or a TATA box. The promoters may even contain other sequences to affect (such as to maintain, enhance, decrease) the levels of expression of the nucleotide sequence of the present invention. For example, suitable other sequences include the *Sh1*-intron or an ADH intron. Other sequences include inducible elements - such as temperature, chemical, light or stress inducible elements.

Also, suitable elements to enhance transcription or translation may be present. An example of the latter element is the TMV 5' signal sequence (see Sleat Gene 217 [1987] 217-225; and Dawson Plant Mol. Biol. 23 [1993] 97).

Thus, in one aspect, the nucleotide sequence according to the present invention is under the control of a promoter that allows expression of the nucleotide sequence. In this aspect, the promoter may be a cell or tissue specific promoter. If, for example, the organism is a plant then the promoter can be one that affects expression of the nucleotide sequence in any one or more of seed, stem, tuber, sprout, root and leaf tissues.

General teachings of recombinant DNA techniques may be found in Sambrook,J., Fritsch, E.F., Maniatis T. (Editors) Molecular Cloning. A laboratory manual. Second edition. Cold Spring Harbour Laboratory Press. New York 1989.

Even though the enzyme and the nucleotide sequence of the present invention are not disclosed in EP-B-0470145 and CA-A-2006454, those two documents do provide some useful background commentary on the types of techniques that may be employed to prepare transgenic plants according to the present invention. Some of these background teachings are now included in the following commentary.

The basic principle in the construction of genetically modified plants is to insert genetic information in the plant genome so as to obtain a stable maintenance of the inserted genetic material.

Several techniques exist for inserting the genetic information, the two main principles being direct introduction of the genetic information and introduction of the genetic information by use of a vector system. A review of the general techniques may be found in articles by Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27).

Thus, in one aspect, the present invention relates to a vector system which carries the nucleotide sequence or construct according to the present invention and which is capable of introducing the nucleotide sequence or construct into the genome of an organism, such as a plant.

The vector system may comprise one vector, but it can comprise two vectors. In the case of two vectors, the vector system is normally referred to as a binary vector system. Binary vector systems are described in further detail in Gynheung An et al. (1980), Binary Vectors, *Plant Molecular Biology Manual A3*, 1-19.

One extensively employed system for transformation of plant cells with a given promoter or nucleotide sequence or construct is based on the use of a Ti plasmid from *Agrobacterium tumefaciens* or a Ri plasmid from *Agrobacterium rhizogenes* An et al. (1986), *Plant Physiol.* 81, 301-305 and Butcher D.N. et al. (1980), *Tissue Culture Methods for Plant Pathologists*, eds.: D.S. Ingrams and J.P. Helgeson, 203-208.

Several different Ti and Ri plasmids have been constructed which are suitable for the construction of the plant or plant cell constructs described above.

The nucleotide sequence or construct of the present invention should preferably be inserted into the Ti-plasmid between the terminal sequences of the T-DNA or adjacent a T-DNA sequence so as to avoid disruption of the sequences immediately surrounding the T-DNA borders, as at least one of these regions appear to be essential for insertion of modified T-DNA into the plant genome.

As will be understood from the above explanation, if the organism is a plant, then the vector system of the present invention is preferably one which contains the sequences necessary to infect the plant (e.g. the *vir* region) and at least one border part of a T-DNA sequence, the border part being located on the same vector as the genetic construct.

Furthermore, the vector system is preferably an *Agrobacterium tumefaciens* Ti-plasmid or an *Agrobacterium rhizogenes* Ri-plasmid or a derivative thereof, as these plasmids are well-known and widely employed in the construction of transgenic plants, many vector systems exist which are based on these plasmids or derivatives thereof.

In the construction of a transgenic plant the nucleotide sequence or construct of the present invention may be first constructed in a microorganism in which the vector can replicate and which is easy to manipulate before insertion into the plant. An example of a useful microorganism is *E. coli*, but other microorganisms having the above properties may be used. When a vector of a vector system as defined above has been constructed in *E. coli*, it is transferred, if necessary, into a suitable *Agrobacterium* strain, e.g. *Agrobacterium tumefaciens*. The Ti-plasmid harbouring the nucleotide sequence or construct of the invention is thus preferably transferred into a suitable *Agrobacterium* strain, e.g. *A. tumefaciens*, so as to obtain an *Agrobacterium* cell harbouring the nucleotide sequence or construct of the invention, which DNA is subsequently transferred into the plant cell to be modified.

As reported in CA-A-2006454, a large amount of cloning vectors are available which contain a replication system in *E. coli* and a marker which allows a selection of the transformed cells. The vectors contain for example pBR 322, pUC series, M13 mp series, pACYC 184 etc. In this way, the nucleotide or construct of the present invention can be introduced into a suitable restriction position in the vector. The contained plasmid is used for the transformation in *E.coli*. The *E.coli* cells are cultivated in a suitable nutrient medium and then harvested and lysed. The plasmid is then recovered. As a method of analysis there is generally used sequence analysis, restriction analysis, electrophoresis and further biochemical-molecular biological methods. After each manipulation, the used DNA sequence can be restricted and connected with the next DNA sequence. Each sequence can be cloned in the same or different plasmid.

After each introduction method of the construct or nucleotide sequence according to the present invention in the plants the presence and/or insertion of further DNA sequences may be necessary. If, for example, for the transformation the Ti- or Ri-plasmid of the plant cells is used, at least the right boundary and often however the right and the left boundary of the Ti- and Ri-plasmid T-DNA, as flanking areas of the introduced genes, can be connected. The use of T-DNA for the transformation of plant cells has been intensively studied and is described in EP-A-120516; Hoekema, in: The Binary Plant Vector System Offset-drukkerij Kanters B.B., Alblasserdam, 1985, Chapter V; Fraley, et al., Crit. Rev. Plant Sci., 4:1-46; and An et al., EMBO J. (1985) 4:277-284.

Direct infection of plant tissues by *Agrobacterium* is a simple technique which has been widely employed and which is described in Butcher D.N. et al. (1980), *Tissue Culture Methods for Plant Pathologists,* eds.: D.S. Ingrams and J.P. Helgeson, 203-208. For further teachings on this topic see Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27). With this technique, infection of a plant may be done on a certain part or tissue of the plant, i.e. on a part of a leaf, a tuber, a root, a stem or another part of the plant.

Typically, with direct infection of plant tissues by *Agrobacterium* carrying the nucleotide sequence of the present invention, a plant to be infected is wounded, e.g. by cutting the plant with a razor or puncturing the plant with a needle or rubbing the plant with an abrasive. The wound is then inoculated with the *Agrobacterium*. The inoculated plant or plant part is then grown on a suitable culture medium and allowed to develop into mature plants.

When plant cells are constructed, these cells may be grown and maintained in accordance with well-known tissue culturing methods such as by culturing the cells in a suitable culture medium supplied with the necessary growth factors such as amino acids, plant hormones, vitamins, etc.

Regeneration of the transformed cells into genetically modified plants may be accomplished using known methods for the regeneration of plants from cell or tissue cultures, for example by selecting transformed shoots using an antibiotic and by subculturing the shoots on a medium containing the appropriate nutrients, plant hormones, etc.

Even further useful teachings on the transformation of plants can be found in Danish patent application No. 940662 (filed 10 June 1994) and/or United Kingdom patent application No. 9702592.8 (filed 7 February 1997).

Reference may even be made to Spngstad *et al* (1995 Plant Cell Tissue Organ Culture **40** pp 1-15) as these authors present a general overview on transgenic plant construction.

In summation, the present invention relates to an enzyme having $\alpha(1,4)$ glucan acetyl-transferase activity and a nucleotide coding for same. The present invention also provides a modified carbohydrate (preferably starch) obtainable from use of the same.

The following sample was deposited in accordance with the Budapest Treaty at the recognised depositary The National Collections of Industrial and Marine Bacteria

Limited (NCIMB) at 23 St. Machar Drive, Aberdeen, Scotland, United Kingdom, AB2 1RY on 7 March 1996:

DH5α-pMAC3 (which contains a 3.2 kb *EcoR*I-*Pst*1 fragment from *E.coli* comprising the *mac* gene).

The deposit number is NCIMB 40789.

This deposit concerns the plasmid pMAC3.

The following sample was deposited in accordance with the Budapest Treaty at the recognised depositary The National Collections of Industrial and Marine Bacteria Limited (NCIMB) at 23 St. Machar Drive, Aberdeen, Scotland, United Kingdom, AB2 1RY on 7 March 1996:

NF1830-pMAC5 (which contains the *E.coli mac* gene).

The deposit number is NCIMB 40790.

This deposit concerns the plasmid pMAC5.

A highly preferred aspect of the present invention therefore relates to an enzyme having α(1,4) glucan acetyl-transferase activity, wherein the enzyme comprises the amino acid sequence shown as SEQ ID No. 1, or a variant, homologue or fragment thereof; and wherein the enzyme is expressed by a nucleotide sequence obtainable from either deposit number NCIMB 40789 or deposit number NCIMB 40790.

Another highly preferred aspect of the present invention therefore relates to a nucleotide sequence comprising the sequence shown as SEQ ID No. 2, or a variant, homologue or fragment thereof or a sequence that is complementary thereto, and wherein the nucleotide sequence is obtainable from either deposit number NCIMB

40789 or deposit number NCIMB 40790.

The present invention also provides a modified carbohydrate (preferably starch) obtainable from use of this same plasmid.

The present invention will now be described only by way of example in which reference is made to the following Figures:

Figure 1 which shows the nucleotide sequence corresponding to SEQ ID No. 2;

Figure 2 which shows the amino acid sequence corresponding to SEQ ID No. 1;

Figure 3 which shows a nucleotide sequence comprising the sequence corresponding to SEQ ID No. 2;

Figure 4 which is a plasmid map of pMAC1;

Figure 5 which is a plasmid map of pMAC2;

Figure 6 which is a plasmid map of pMAC3;

Figure 7 which is a plasmid map of pMAC5;

Figure 8 which is a plasmid map of pMAC8;

Figure 9 which is a plasmid map of pMAC9; and

Figure 10 which is a plasmid map of pMAC10.

Some details on the Figures are as follows:

Figure 1

Nucleotide sequence corresponding to Seq ID No 2

Figure 2

Amino acid sequence corresponding to Seq ID No 1

183 amino acids

20073 MW

Figure 4

Plasmid name: pMAC1

Plasmid size: 7.26 kb

Comments: Insertion of a 4.3 kb *EcoR*1 fragment from *lambda* 151 into the *EcoR*1 site of pBluescript II SK +.

Figure 5

Plasmid name: pMAC2

Plasmid size: 7.26 kb

Comments: Insertion of a 4.3 kb *EcoR*1 fragment from *lambda* 151 (Kohara collection) into the *EcoR*1 site of pBluescript II SK +.

Figure 6

Plasmid name: pMAC3

Plasmid size: 7.26 kb

Comments: Deletion of the 1.1 kb *Pst*1 fragment from pMAC2.

Figure 7

Plasmid name: pMAC5

Plasmid size: 4060 bp

Comments:

The *E coli mac* gene was amplified with primers:

#B411 (upper primer with *EcoR*1 site)

CGG AAT TCC GCC ATG AAG ACA TAC CC

#B412 (lower primer with *Hind*III site)

CAC AAG CTT ATT TTG CAT AAC AGT TGC

using pMAC3 as template.

The 704 bp PCR product was digested with *EcoR*1 and *Hind*III and inserted in pUHE21-2 digested with the same restriction enzymes.

Figure 8

Plasmid Name: pMAC8

Plasmid size: 4935 bp

Comments: The *E coli mac* gene was amplified with primers

B 478 CGG GAT CCG AGC ACA GAA AAA GAA AAG ATG (upper primer with *BamH*I site)

B 479 AAC TGC AGA TTT TGC ATA ACA GTT GC (lower primer with *Pst*I site)

and pMAC3 as template. The PCR product was digested with *BamH*I and *Pst*I and inserted in pBETP5 digested with the same enzymes.

The SBE TP-*mac* fusion was control sequenced with primer # C028

The 35S terminator-*mac* fusion was sequenced with primer # B456 og # C027.

Figure 9

Plasmid name: pMAC9

Plasmid size: 9.37 kb

Comments: Insertion of the 2294 bp *EcoR*I fragment (Patatin promoter-SBE TP-*mac* - 35S terminator) from pMAC8 in the EcoRI site of pVictor IV Man.

Figure 10

Plasmid name: pMAC10

Plasmid size: 9.37 kb

Comments: Insertion of a 2294 bp *EcoR*1 fragment (Patatin promoter-SBE TP-mac-35S terminator) from pMAC8 in the *EcoR*1 site of pVictor IV Man.

Cloning and sequencing of the *mac* gene from *E. coli.*

Following, initially the teachings of Boos and Brand (3), the *mac* gene was isolated from the 4.3 kb *EcoR*I fragment from λ phage 8C4 (151) from the Kohara collection (4). The fragment was inserted into the *EcoR*I site of plasmid pBluescript II SK (+) in both orientations yielding plasmids pMAC1 and pMAC2 (Figures 4 and 5). When harboured in *E. coli* these plasmids gave rise to highly elevated maltose acetyltransferase levels indicating that the 4.3 kb *EcoR*I fragment contains the *mac* gene.

In order to localise the *mac* gene on the 4.3 kb *EcoR*I fragment, the 1.1 kb *Pst*I fragment was deleted from plasmid pMAC2. This plasmid construction pMAC3 (Figure 6) also gave rise to increased maltose acetyltransferase levels in strains containing this plasmid, thus demonstrating that the *mac* gene is present on the 3.2 kb *EcoR*I-*Pst*I fragment.

The nucleotide sequence of the 3.2 kb *EcoR*I-*Pst*I insert in pMAC3 was then determined by automated sequencing on an A.L.F. sequencer. The 3137 bp DNA sequence revealed a 372 bp region of the 3' end of the *E. coli acrB* gene and three open reading frames potentially encoding proteins of 124, 126, and 183 amino acids (Figure 3).

In accordance, ^{35}S-methionine labelling experiments with *E. coli* minicells containing pMAC3 showed the synthesis of proteins having molecular weights corresponding to these sizes.

The 183 codon orf which encodes a protein of a predicted molecular weight of 20073 (Figure 2) is the *mac* gene, since the *E. coli* maltose acetyl-transferase has an estimated subunit molecular weight of 20.000 (3).

Over-expression of the Mac enzyme in *E. coli*.

In order to purify the Mac enzyme, the *mac* gene was inserted after an isopropylthiogalactosidase (IPTG) inducible phage T7-promoter A1 in pUHE21-2 to give pMAC5 (Figure 7). Cultures of *E coli* strain NF1830 (MC1000, recA1, F' lacIq1Z::tm5, a gift from Niels Fiil, University of Copenhagen) harbouring pMAC5 was found to have highly elevated levels of maltose acetyltransferase, when expression of the *mac* gene is induced by addition of IPTG to the growth medium.

Growth Conditions

A 1 L LB culture of NF1830-pMAC5 supplemented with ampicillin (100 μg/ml) and kanamycin (25 μg/ml) was grown at 37°C with vigorous shaking until the A600 reached 0.7. IPTG was added to a final concentration of 2mM and growth was continued for four hours. The cells were harvested by centrifugation (10 min. at 4 000 x g) and washed by resuspension in 200 ml 0.9% NaCl. The cell pellet was then resuspended in 250 ml 20 mM potassium phosphate pH 7.5 containing 0.4 mM PMSF, 0.4 mg/ml pepstatin and 1.6 mM EDTA. The suspension was sonicated 5 x 1 min. using a Vibra Cell VC 600 with a 19 mm High Gain Horn and extender (all from Sonics and Materials Inc., USA). The homogenate was clarified by centrifugation for 60 min. at 90 000 x g at 4°C and subsequent filtration through a 0.22 μm filter.

Purification of Recombinant Mac

The resulting crude extract was applied to a Q-Sepharose 26/10 column (Pharmacia Biotech) equilibrated with 20 mM potassium phosphate pH 7.5 (hereinafter called "buffer A") at a flow rate of 2 ml/min. The column was washed with 300 ml of buffer A and the bound protein was eluted by applying a 0 to 0.3 M NaCl linear gradient in buffer A (300 ml). The fractions containing enzyme activity were pooled and applied to a 8 ml Affi-Gel Blue (Biorad) column (16 mm x 26 cm) equilibrated with buffer A at a flow rate of 1 ml/min. The column was washed with 50 ml of the same buffer containing 0.4 M NaCl. The enzyme was then eluted with the same buffer containing 2 M NaCl. The active pool was dialysed overnight against buffer A and subsequently concentrated to approximately 3 ml in a Centriprep-30 (Amicon). This fraction was applied to a 6 ml Acetyl-coA-Minileak column equilibrated with buffer A at a flow rate of 0.3 ml/min. This affinity resin was made by coupling 200 mg of Acetyl-coA to 5 g (dry weight) of Minileak High (Kem-En-Tek, Denmark) in 10 ml of 1 M $NaCO_3$ pH 11 for 20h at room temperature. The column was washed with 20 ml of buffer A. It was then turned upside down and the pure enzyme was eluted in less than 20 ml with buffer A containing 0.5 M NaCl.

The purification of the maltose acetyltransferase to homogeneity was achieved after three chromatographic steps. From 1l culture we were able to get 5.8 mg pure Mac. The yield was 29% and the enzyme was purified 80-fold. The purity of the enzyme was assessed both by SDS-PAGE and mass spectrometry. The latter revealed a molecular mass of 19,982 Da.

Determination of enzyme concentration and activity

The concentration of pure Mac solutions was estimated spectrophotometrically at 280 nm using an extinction coefficient of 0.66 as determined from the amino acid composition of Mac according to (5). The acetyl-transferase activity of Mac was assayed spectrophotometrically according to a modified Alpers' assay (6). A Perkin Elmer Lambda 18 spectrophotometer was used. The assay mixture of a total volume of 1 ml contained a 50 mM potassium phosphate, 2 mM EDTA buffer at pH 7.5, 100 μl of maltose 1M, 100 μl of Acetyl-coA 0.4 mM, 10 μl 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) 40 mM dissolved in methanol and 10 μl enzyme. The reaction was started by the addition of enzyme or maltose and was monitored at 412 nm at 25°C. One activity unit was defined as the amount of enzyme that produced an increase in absorbance of 1 per minute at 25°C. An extinction coefficient of 13 600 M^{-1} x cm^{-1} was used for DTNB in order to calculate the consumption of acetyl coenzyme A.

N-terminal sequencing of Recombinant Mac

N-terminal sequencing of pure Mac was performed using an Applied Biosystems 476A protein sequencer. One nanomole of protein was desalted by RP-HPLC on a C2 column (4.6/30) prior to loading onto the sequencer. The N-terminal sequence of Mac was determined up to residue was determined up to residue 48 and was in complete concordance with the nucleotide sequence of the *mac* gene (Figure 1). Furthermore, the N-terminal methionine residue was not present on the mature protein (Figure 2).

Production of polyclonal antibodies against Recombinant Mac

Rabbits were immunised subcutaneously at 2-week intervals during 6 weeks and at 4-week intervals thereafter with 90 μg of pure protein emulsified (1:1, vol/vol) with Freund's adjuvant. Antisera were tested against Mac in immunoblots and were found highly specific.

Characterisation and activity profile of recombinant Mac

Mass spectrometry studies indicated that Mac may be a trimer.

The isoelectric point of Mac was determined by isoelectric focusing on a PhastGel IEF 4-6.5 (Pharmacia) and was found to be 5.7.

The pH profile of Mac was investigated between pH 5 and 8.5 at a 100 mM maltose concentration in 50 mM buffers containing 100 mM NaCl. Under these conditions, the pH optimum was 7.7.

The pH stability of Mac was examined at 25°C between pH 3.0 and 10.0. Mac was instantaneously inactivated at pH 3.0 but was stable between pH 4.0 and 10.0 for at least six hours.

The thermostability of Mac was investigated at pH 7.5 between 40 and 70°C. After incubation for four hours at 40°C and 50°C, the remaining activity of Mac was 100% and 75%, respectively. Its half-life was 70 min, and 22 min at 60°C and 70°C, respectively.

The substrate preference of Mac towards the carbohydrate acetyl-acceptor substrate was investigated by measuring the initial rate of the acetylation of various carbohydrates (used at 50 and 100 mM concentrations) following the procedure described in "Determination of enzyme concentration and activity". The results are presented in Tables 1, 2 and 3. Among the monosaccharides tested, glucose was the best substrate and among the disaccharides tested, maltose and isomaltose were the best substrates.

Table 1. Comparison of the relative activity of Mac towards various monosaccharides as acetyl-acceptors.

Substrate (100 mM)	Relative Activity (% of activity on glucose)
Glucose	100
Mannose	38
Fructose	17
Galactose	0.9

Table 2. Comparison of the relative activity of Mac towards various disaccharides as acetyl-acceptors.

Substrate (100 mM)	Relative Activity (% of activity on maltose)
Maltose (α-glucose(1,4) α-glucose)	100
Isomaltose (α-glucose(1,4) α-glucose)	110
Lactose (β-galactose β-(1,6) α-glucose)	0.4
Sucrose (α-glucose α-(1,4) β-fructose)	0.4
Cellobiose (β-glucose β-(1,4) β-glucose)	0

Table 3. Comparison of the relative activity of Mac towards various maltooligosaccharides as acetyl-acceptors.

Substrate (50 mM)	Relative Activity (% of activity on maltose)
Maltose	100
Maltotriose	7.5
Maltotetraose	0.5
Maltopentaose	0.9
Maltohexaose	1.2
Maltoheptaose	1.1

Kinetic studies

Kinetic studies of Mac catalysed acetylation reactions revealed that the Km for the acceptor substrate is in the mM range whereas it is in the μM range for acetyl-coenzyme A. Thus, Mac has about a 1000 fold more affinity for acetyl-coenzyme A than for the acceptor.

NMR studies

^{1}H-NMR structure determination of the products of acetylation of glucose and maltose by Mac was investigated.

In order to investigate the substrate regio-specificity of Mac regarding the acetylation site of the acceptor substrate, we prepared milligram amounts of acetylated glucose and maltose by incubating 10 mg of glucose or maltose with *E. coli* Mac and 1 mg acetyl-coenzyme A in phosphate buffer at pH 7.5 for 48 hours. Additional aliquots

of 1 mg acetyl-coenzyme A were added during the incubation. The reaction products were separated by thin layer chromatography and the acetylated glucose and maltose were isolated from the chromatogram and freeze dried. The structures of these acetylated sugars were determined by ^{1}H-NMR. Glucose was only acetylated at the C6 position, and maltose was acetylated at the C6 position of its non-reducing glucose moiety. These results reveal that Mac acetylates hexoses at their C6 position.

Activity of the SBE-Mac fusion in *E. coli*.

Because the 27 amino acid SBE portion of the SBE-Mac fusion in pMAC9 and pMAC10 described below may interfere with the acetyltransferase activity, the SBE-Mac fusion was inserted in the *E. coli* expression vector pAL781 (Invitrogene, San Diego, USA) in order to over-express the fusion enzyme in *E. coli* and analyse the activity. A comparison of the highly over-expressed SBE-Mac fusion and the purified wild type Mac enzyme on SDS gels showed that the fusion migrated slightly slower due to the 27 amino acid extension. Moreover, the fusion retained the ability to use maltose as a substrate for acetylation. Thus, the fusion enzyme appears to be intact and is fully active in *E. coli*. Therefore, it may be assumed, that the SBE-Mac fusion enzyme will be active in potatoes.

IN VIVO MODIFICATION OF STARCH IN POTATO

General teachings on potato transformation may be found in our copending patent applications PCT/EP96/03053, PCT/EP96/03052 and PCT/EP94/01082 (the contents of each of which are incorporated herein by reference).

For the present studies, the following protocol was adopted.

Construction of plasmids for the expression of the *E coli mac* gene in potato.

The *E coli mac* gene was amplified with primers:

5'-CGG GAT CCG AGC ACA GAA AAA GAA AAG ATG-3' (upper primer with *BamH*I site)

and

5'-AAC TGC AGA TTT TGC ATA ACA GTT GC-3' (lower primer with *Pst*I site)

and pMAC3 as template.

The PCR product was digested with *BamH*I and *Pst*I and inserted in pBETP5 (see PCT patent application No. WO 94/24292, the contents of which are incorporated herein by reference) digested with the same enzymes yielding pMAC8. Thereby, the *mac* gene is inserted in an expression cassette that provides tuber specific expression from a patatin promoter and transcription termination at a CaMV 35S terminator. Moreover, the Mac enzyme is fused to 102 amino acids of the N-terminus of the potato starch branching enzyme including a 75 amino acid transit peptide that directs the *mac* gene product to the potato tuber amyloplasts. Upon import to the amyloplast the 75 amino acid transit peptide is cleaved off, to give a Mac fusionprotein that has the 27 amino acids from the mature starch branching enzyme N-terminus. The 2294 bp *EcoR*I expression cassette was isolated from pMAC8 and inserted in the *EcoR*I site of the plant transformation vector pVictor IV Man (see PCT patent application No. WO 94/24292 and British patent application No. 951443.8, the contents of each of which are incorporated herein by reference) giving plasmids pMAC9 and pMAC10 (Figures 9 and 10, respectively).

Preparation of potato minitubers

A segment containing the nodium - i.e. a segment taken from 2 mm above and 5 mm below the nodium - was cut from *in vitro* grown potato plants or mannose selected shoots (for mannose selection see our earlier patent applications WO 93/05163 and/or WO 94/20627). The leaf was removed from the nodium segment, and the segment was placed vertically on agar plates with MS medium (Sigma) supplemented with 60 g sucrose/l and 2 mg 6-benzyl-aminopurine/l. The nodium segments were grown for 7 days at 20°C with a 16 hour light period and an 8 hour dark period. Subsequently, the plates were wrapped in alu-foil and placed in the dark at 20°C. The minitubers were harvested after about 28 days and applied for western analysis in order to detect Mac expression.

Expression of the SBE-Mac fusion in potato minitubers

Potato minitubers transformed with the pMAC9 or pMAC10 constructs were examined by Western analysis for expression of the *E. coli* mac gene with antibodies raised towards the *E. coli* maltose acetyltransferase. The analysis clearly demonstrated that 3 out of 5 MAC9 minitubers and 5 out of 7 MAC10 minitubers gave a distinct expression of the *E. coli* maltose acetyltransferase. The positive minitubers expressed a 209 amino acid SBE-Mac fusion that co-migrates with a similar construction expressed in *E. coli*. These results indicate that the 75 amino acid SBE transit peptide, that was originally fused to the 209 amino acid SBE-Mac fusion, has been removed from the SBE-fusion. Furthermore, this implies that the transit peptide was correctly processed by the signal peptidase in the amyloplast membrane, and that the SBE-Mac fusion has been directed to the amyloplast.

Immunoblots on potato tuber extracts

0.5 ml potato protein extract was precipitated with 20% TCA for 30 min on ice. Protein precipitates were recovered after centrifugation and resuspended in 50 µl of SDS-PAGE sample buffer. 25 µl were subsequently loaded onto 15 % polyacrylamide gels. After electrophoresis proteins were transferred onto Problot PVDF membranes by semi-dry blotting. For immunodetection Mac antiserum was diluted 1:2 000 and secondary antibody was coupled to alkaline phosphatase.

In accordance with the Western Blot analysis of the minitubers described above, the western analysis of the transgenic tubers clearly demonstrated that the 209 a SBE-Mac fusion is expressed in the tubers.

Analysis of potato tubers for Mac activity

Potato tubers of comparable sizes were chosen and cut into pieces and homogenised in extraction buffer and Dowex (1%, w/vol) using a mortar and pestle or an electric blender. 5 ml extraction buffer (50 mM potassium phosphate pH 7.5, 2 mM EDTA, 0.5 mM PMSF) was used per gram potato. The mixture was allowed to stand on ice for 30 min and the insoluble material was removed by centrifugation. Protein concentration was measured using the BCA reagent (Pierce).

Mac activity was measured in duplicates or triplicates as follows: 0, 50, 100 or 200 µl potato extract, 10 µl of 1 mM acetyl-coenzyme A, 25 µl of 1 M glucose and assay buffer (50 mM potassium phosphate, 2 mM EDTA, pH 7.5) were mixed per microtiter plate well to give a total volume of 250 µl. The reaction was started by the addition of acetyl-coenzyme A. After 10 min. reaction at room temperature, 25 µl of freshly made 4 mM DTNB was added and A_{405} was measured immediately. Two wells were prepared for each single assay, one with glucose and one without. Activity was calculated by subtracting the absorbance of the well without glucose (background absorbance) from that of the well with glucose.

Relatively high levels of Mac activity could be measured in eight out of nine transgenic tubers. Some of the tubers had a Mac activity that was 15 to 20 fold above the almost negligible activity found in non-transformed tubers.

Viscometric studies

Samples of starch obtained from tubers of non-transformed potatoes and from transformed potatoes according to the present invention were analysed by viscoamylograph of an aqueous suspension using a Newport Scientific Rapid Visco Analyser 3C. The results showed that the starch from the transformed potatoes had a different viscometric profile to the starch from the non-transformed potato.

DSC studies

Samples of starch obtained from tubers of non-transformed potatoes and from transformed potatoes according to the present invention were analysed by differential scanning colometry (using a 10% w/w aqueous starch suspension). The samples were heated from 20 to 100°C at a velocity of 10°C per minute. The results showed that the starch from the transformed potatoes had a different enthalpy to the starch from the non-transformed potato. We additionally found a difference in gelatinisation temperature for the transformed potatoes compared to the starch from the non-transformed potatoes.

Other modifications of the present invention will be apparent to those skilled in the art.

REFERENCES

1. Boos W., Ferenci T. & Shuman H. A. 1981. *J. Bacteriol.* **146**, 725-732.

2. Freundlieb S. & Boos W. 1982. *Ann. Microbiol. (Inst. Pasteur)* **133 A,** 181-189.

3. Brand B. & Boos W. 1991. *J. Biol. Chem.* **266**, 14113-14118.

4. Kohara et al. 1987. *Cell* **50**: July 31 issue.

5. Gill S. C. & von Hippel P. H. 1989. *Anal. Biochem.* **182**, 319-326.

6. Alpers D. H., Appel S. H. & Tomkrins G. M. 1965. *J. Biol. Chem.* **240**, 10-13.

7. Ogasawara N., Nakai S. & Yoshikawa H. 1994, DNA Research 1, 1-14.

SEQUENCES

SEQUENCE ID NO 1

Amino acid sequence

MSTEKEKMIAGELYRSADETLSRDRLRARQLIHRYNHSLAEEHTLRQQIL 50

ADLFGQVTEAYIEPTFRCDYGYNIFLGNNFFANFDCVMLDVCPIRIGDNC 100

MLAPGVHIYTATHPIDPVARNSGAELGKPVTIGNNVWIGGRAVINPGVTI 150

GDNVVVASGAVVTKDVPDNVVVGGNPARIIKKL 183

SEQUENCE ID NO 2

Nucleotide sequence

ATGAGCACAG AAAAAGAAAA GATGATTGCT GGTGAGTTGT
ATCGCTCGGC AGATGAGACG TTATCTCGCG ATCGCCTGCG
CGCTCGTCAG CTTATTCACC GATACAATCA TTCCCTGGCG
GAAGAGCACA CATTACGCCA GCAAATTCTC GCTGATCTAT
TCGGTCAGGT GACAGAGGCT TATATTGAGC CAACGTTTCG
CTGTGACTAT GGCTATAACA TTTTTCTCGG TAATAATTTT
TTCGCCAACT TCGATTGCGT GATGCTTGAT GTCTGCCCTA
TTCGCATCGG TGATAACTGT ATGTTGGCAC CAGGCGTTCA
TATCTACACG GCAACACATC CCATCGACCC TGTAGCACGT
AATAGCGGTG CTGAACTGGG GAAACCCGTC ACCATCGGTA
ATAACGTCTG GATTGGCGGA CGCGCGGTCA TTAACCCTGG
TGTGACCATT GGTGATAACG TCGTGGTAGC CTCAGGTGCA
GTTGTCACAA AAGATGTCCC GGACAACGTT GTCGTGGGCG
GTAATCCAGC CAGAATAATT AAAAAATTGT AA

SEQUENCE I D NO 3

Nucleotide sequence

Complete nucleotide sequence of the 3.2 kb *EcoRI-PstI* fragment in pMAC3

GAATTCGCCA AAGACTTGAT GGATAAAGAA GGTAAAGGTC TGATTGAAGC GACGCTTGAT 60
GCGGTGCGGA TGCGTTTACG TCCGATCCTG ATGACCTCGC TGGCGTTTAT CCTCGGCGTT 120
ATGCCGCTGG TTATCAGTAC TGGTGCTGGT TCCGGCGCGC AGAACGCAGT AGGTACCGGT 180
GTAATGGGCG GGATGGTGAC CGCAACGGTA CTGGCAATCT TCTTCGTTCC GGTATTCTTT 240
GTGGTGGTTC GCCGCCGCTT TAGCCGCAAG AATGAAGATA TCGAGCACAG CCATACTGTC 300
GATCATCATT GATACAACGT GTAATCACTA AGGCCGCGTA AGCGGCCTTT TTTATGCATA 360
ACCTACGAAC ATTAAGGAGT AATTGAACCA CCAACTCAGG ATCTCATACG AAAACCAGTA 420
TTAACCACGG ATAAAATTCA TAAAAAATAC TGATTGTTAG TTAATTTATA TTAAGTAGCG 480
CTAATAGATT TAATAATCCA TAATCATTTA GAGGCTATTC TTAATTATTT GCGGTAATTC 540
TTTATTCATT CCTCGGTTAT TACGTCATAT TCAGAGCAAT CCTGGTATTA GTGTCACCAA 600
TTTCATCTGG CGATAATCCT GAAATGTTAT GAATAGTTCG AGCAAACTGC TTTTACCTGC 660
TGCGGGTTAG TGCTAGTATG AAAAAGTGAG TCCTGTCCCG CTTCCTTCCT AATTGTAATT 720
TTTCGTAATA ATGCGATGAA AACCTGCAAA GAGTGGCTTA TAGTTAAGCT AACAAACGAG 780
AGGGCAAGTC CAGGTCAGTA AGTTTTTTCC ATCCCGAAAG GTGTCCGTTA GTTCAACCGC 840
TAAGAAGGGG ACGCGTTATG GATGAATACT CACCCAAAAG ACATGATATC GCACAGCTTA 900
AGTTTCTCTG TGAAACCCTG TATCATGACT GCCTTGCAAA CCTTGAAGAA AGCAATCATG 960
GCTGGGTAAA CGACCCAACC TCGGCGATCA ACCTCCAGTT GAATGAACTG ATTGAGCATA 1020
TTGCGACCTT CGCACTTAAT TACAAAATTA AGTATAATGA AGACAATAAG CTCATTGAGC 1080
AGATCGACGA ATATCTGGAT GACACCTTTA TGTTGTTCAG TAGTTATGGT ATTAATATGC 1140
AGGATCTTCA GAAATGGCGG AAGTCAGGTA AHCGACTATH CCGTTGTTTT GTCAATGCGA 1200
CGAAAGAGAA TCCTGCGAGT TTATCTTGTT AGAATTATTA CAACCATAGG TAGAAGTATG 1260
TCCGAAAAAC CTTTAACGAA AACCGATTAT TTAATGCGTT TACGTCGTTG CCAGACAATT 1320
GACACGCTGG AGCGGTTTAW TCGAGAAAAA TAAATACGAA TTATCAGATA ATGAACTGGC 1380
GGTATTTTAC TCAGCCGCAG ATCACCGCCT CGCCGAATTG ACCATGAATA AACTGTACGA 1440
CAAGATCCCT TCCTCAGTAT GGAAATTTAT TCGCTAATAA ATAATTCGCT TTCGGAGCTA 1500
TAACCGGCTG TTTATTAAGA ATTTTATACT TTTTCGCCAT GAAGACATAC CCTATGTGAT 1560
CTTTATCACA CAGATGTAAT GGGAACGTTC TCTTCACTGA CTTTTCGTCT TACTGTGTTG 1620
CCGCATTTTC AGCAACCGGA GTCAGTAATG AGCACAGAAA AAGAAAAGAT GATTGCTGGT 1680
GAGTTGTATC GCTCGGCAGA TGAGACGTTA TCTCGCGATC GCCTGCGCGC TCGTCAGCTT 1740
ATTCACCGAT ACAATCATTC CCTGGCGGAA GAGCACACAT TACGCCAGCA AATTCTCGCT 1800
GATCTATTCG GTCAGGTGAC AGAGGCTTAT ATTGAGCCAA CGTTTCGCTG TGACTATGGC 1860
TATAACATTT TTCTCGGTAA TAATTTTTTC GCCAACTTCG ATTGCGTGAT GCTTGATGTC 1920
TGCCCTATTC GCATCGGTGA TAACTGTATG TTGGCACCAG GCGTTCATAT CTACACGGCA 1980
ACACATCCCA TCGACCCTGT AGCACGTAAT AGCGGTGCTG AACTGGGGAA ACCCGTCACC 2040
ATCGGTAATA ACGTCTGGAT TGGCGGACGC GCGGTCATTA ACCCTGGTGT GACCATTGGT 2100
GATAACGTCG TGGTAGCCTC AGGTGCAGTT GTCACAAAAG ATGTCCCGGA CAACGTTGTC 2160
GTGGGCGGTA ATCCAGCCAG AATAATTAAA AAATTGTAAT CGGTTTTTCG CAACTGTTAT 2220
GCAAAATTGT GGTAGATCTG TTACTTCCCC TCTACTATTC CCACGTTAAA ATAGGGTGTT 2280
CCCTGGAAAG TTGCAGATAC CACGAAGGCA AACGATGACC GAAATACAAC GCCTGCTGAC 2340
CGAAACGATT GAGTCTCTGA ATACCCGCGA AAAACGCGAC AACAAACCCC GCTTTAGTAT 2400

CAGTTTTATC CGTAAACATC CGGGGCTGTT TATCGGTATG TACGTTGCTT TTTTTGCCAC 2460
CCTGGCGGTG ATGTTGCAGT CCGAAACGCT GTCAGGCTCT GTCTGGCTAC TGGTTGTATT 2520
ATTTATCCTG CTTAATGGTT TCTTCTTTTT CGATGTCTAC CCACGCTACC GCTATGAAGA 2580
TATCGACGTG CTGGATTTCC GCGTTTGCTA TAACGGCGAA TGGTACAACA CGCGCTTTGT 2640
ACCTGCCGCG CTGGTTGAAG CCATCTTGAA CTCTCCGTGT CGCGGATGTT CATAAGGAAC 2700
AACTGCAAAA AATGATCGTC CGTAAAGGTG AACTGTCTTT TTACGATATT TTTACCCTCS 2760
TCGCGCCGAA TCAACATCTT AAGTTAGGGT TACATACCAG GCGTAAAGCT CTGCGCCTGG 2820
TCAAATGACA ATGATCGTTT CCACCCATCA CTTCATGAAA TACCAGCTCT ACCTCCTTAT 2880
CTCCAGCCAG CCTTTTTCCA CAATCAGATA TACTTTCCCT ACACTGTGTT AATAAGGATA 2940
TGCTGGTGAG AACACGACAT CTGGTCGGCC TTATTTCGGG AGTACTGATT CTTTCAGTAT 3000
TGCTGCCTGT CGGCTTAAGC ATCTGGCTGG CCCATCAGCA GGTAGAAACA TCGTTTATTG 3060
AAGAGCTGGA TACCTATTCC TCCCGCGTCG CTATTCGAGC CAATAAGGTG GCGACACAAG 3120
GGAAAGATGC GCTGCAG 3137

SEQUENCE I.D. NO. 4

Complete nucleotide sequence of the 3.2 kb *EcoRI-PstI* fragment in pMAC3. The Mac enzyme amino acid sequence is also shown below the *mac* gene coding sequence.

GAATTCGCCAAAGACTTGATGGATAAAGAAGGTAAAGGTCTGATTGAAGCGACGCTTGAT 60
GCGGTGCGGATGCGTTTACGTCCGATCCTGATGACCTCGCTGGCGTTTATCCTCGGCGTT 120
ATGCCGCTGGTTATCAGTACTGGTGCTGGTTCCGGCGCGCAGAACGCAGTAGGTACCGGT 180
GTAATGGGCGGGATGGTGACCGCAACGGTACTGGCAATCTTCTTCGTTCCGGTATTCTTT 240
GTGGTGGTTCGCCGCCGCTTTAGCCGCAAGAATGAAGATATCGAGCACAGCCATACTGTC 300
GATCATCATTGATACAACGTGTAATCACTAAGGCCGCGTAAGCGGCCTTTTTTATGCATA 360
ACCTACGAACATTAAGGAGTAATTGAACCACCAACTCAGGATCTCATACGAAAACCAGTA 420
TTAACCACGGATAAAATTCATAAAAAATACTGATTGTTAGTTAATTTATATTAAGTAGCG 480
CTAATAGATTTAATAATCCATAATCATTTAGAGGCTATTCTTAATTATTTGCGGTAATTC 540
TTTATTCATTCCTCGGTTATTACGTCATATTCAGAGCAATCCTGGTATTAGTGTCACCAA 600
TTTCATCTGGCGATAATCCTGAAATGTTATGAATAGTTCGAGCAAACTGCTTTTACCTGC 660
TGCGGGTTAGTGCTAGTATGAAAAAGTGAGTCCTGTCCCGCTTCCTTCCTAATTGTAATT 720
TTTCGTAATAATGCGATGAAAACCTGCAAAGAGTGGCTTATAGTTAAGCTAACAAACGAG 780
AGGGCAAGTCCAGGTCAGTAAGTTTTTTCCATCCCGAAAGGTGTCCGTTAGTTCAACCGC 840
TAAGAAGGGGACGCGTTATGGATGAATACTCACCCAAAAGACATGATATCGCACAGCTTA 900
AGTTTCTCTGTGAAACCCTGTATCATGACTGCCTTGCAAACCTTGAAGAAAGCAATCATG 960
GCTGGGTAAACGACCCAACCTCGGCGATCAACCTCCAGTTGAATGAACTGATTGAGCATA 1020
TTGCGACCTTCGCACTTAATTACAAAATTAAGTATAATGAAGACAATAAGCTCATTGAGC 1080
AGATCGACGAATATCTGGATGACACCTTTATGTTGTTCAGTAGTTATGGTATTAATATGC 1140
AGGATCTTCAGAAATGGCGGAAGTCAGGTAAHCGACTATHCCGTTGTTTTGTCAATGCGA 1200
CGAAAGAGAATCCTGCGAGTTTATCTTGTTAGAATTATTACAACCATAGGTAGAAGTATG 1260
TCCGAAAAACCTTTAACGAAAACCGATTATTTAATGCGTTTACGTCGTTGCCAGACAATT 1320
GACACGCTGGAGCGGTTTAWTCGAGAAAAATAAATACGAATTATCAGATAATGAACTGGC 1380
GGTATTTTACTCAGCCGCAGATCACCGCCTCGCCGAATTGACCATGAATAAACTGTACGA 1440
CAAGATCCCTTCCTCAGTATGGAAATTTATTCGCTAATAAATAATTCGCTTTCGGAGCTA 1500
TAACCGGCTGTTTATTAAGAATTTTATACTTTTTCGCCATGAAGACATACCCTATGTGAT 1560

```
CTTTATCACACAGATGTAATGGGAACGTTCTCTTCACTGACTTTTCGTCTTACTGTGTTG   1620
CCGCATTTTCAGCAACCGGAGTCAGTAATGAGCACAGAAAAAGAAAAGATGATTGCTGGT   1680
                              M  S  T  E  K  E  K  M  I  A  G
GAGTTGTATCGCTCGGCAGATGAGACGTTATCTCGCGATCGCCTGCGCGCTCGTCAGCTT   1740
E  L  Y  R  S  A  D  E  T  L  S  R  D  R  L  R  A  R  Q  L
ATTCACCGATACAATCATTCCCTGGCGGAAGAGCACACATTACGCCAGCAAATTCTCGCT   1800
I  H  R  Y  N  H  S  L  A  E  E  H  T  L  R  Q  Q  I  L  A
GATCTATTCGGTCAGGTGACAGAGGCTTATATTGAGCCAACGTTTCGCTGTGACTATGGC   1860
D  L  F  G  Q  V  T  E  A  Y  I  E  P  T  F  R  C  D  Y  G
TATAACATTTTTCTCGGTAATAATTTTTTTCGCCAACTTCGATTGCGTGATGCTTGATGTC   1920
Y  N  I  F  L  G  N  N  F  F  A  N  F  D  C  V  M  L  D  V
TGCCCTATTCGCATCGGTGATAACTGTATGTTGGCACCAGGCGTTCATATCTACACGGCA   1980
C  P  I  R  I  G  D  N  C  M  L  A  P  G  V  H  I  Y  T  A
ACACATCCCATCGACCCTGTAGCACGTAATAGCGGTGCTGAACTGGGGAAACCCGTCACC   2040
T  H  P  I  D  P  V  A  R  N  S  G  A  E  L  G  K  P  V  T
ATCGGTAATAACGTCTGGATTGGCGGACGCGCGGTCATTAACCCTGGTGTGACCATTGGT   2100
I  G  N  N  V  W  I  G  G  R  A  V  I  N  P  G  V  T  I  G
GATAACGTCGTGGTAGCCTCAGGTGCAGTTGTCACAAAAGATGTCCCGGACAACGTTGTC   2160
D  N  V  V  V  A  S  G  A  V  V  T  K  D  V  P  D  N  V  V
GTGGGCGGTAATCCAGCCAGAATAATTAAAAAAATTGTAATCGGTTTTTCGCAACTGTTAT   2220
V  G  G  N  P  A  R  I  I  K  K  L
GCAAAATTGTGGTAGATCTGTTACTTCCCCTCTACTATTCCCACGTTAAAATAGGGTGTT   2280
CCCTGGAAAGTTGCAGATACCACGAAGGCAAACGATGACCGAAATACAACGCCTGCTGAC   2340
CGAAACGATTGAGTCTCTGAATACCCGCGAAAAACGCGACAACAAACCCCGCTTTAGTAT   2400
CAGTTTTATCCGTAAACATCCGGGGCTGTTTATCGGTATGTACGTTGCTTTTTTTGCCAC   2460
CCTGGCGGTGATGTTGCAGTCCGAAACGCTGTCAGGCTCTGTCTGGCTACTGGTTGTATT   2520
ATTTATCCTGCTTAATGGTTTCTTCTTTTTCGATGTCTACCCACGCTACCGCTATGAAGA   2580
TATCGACGTGCTGGATTTCCGCGTTTGCTATAACGGCGAATGGTACAACACGCGCTTTGT   2640
ACCTGCCGCGCTGGTTGAAGCCATCTTGAACTCTCCGTGTCGCGGATGTTCATAAGGAAC   2700
AACTGCAAAAAATGATCGTCCGTAAAGGTGAACTGTCTTTTTACGATATTTTTACCCTCS   2760
TCGCGCCGAATCAACATCTTAAGTTAGGGTTACATACCAGGCGTAAAGCTCTGCGCCTGG   2820
TCAAATGACAATGATCGTTTCCACCCATCACTTCATGAAATACCAGCTCTACCTCCTTAT   2880
CTCCAGCCAGCCTTTTTCCACAATCAGATATACTTTCCCTACACTGTGTTAATAAGGATA   2940
TGCTGGTGAGAACACGACATCTGGTCGGCCTTATTTCGGGAGTACTGATTCTTTCAGTAT   3000
TGCTGCCTGTCGGCTTAAGCATCTGGCTGGCCCATCAGCAGGTAGAAACATCGTTTATTG   3060
AAGAGCTGGATACCTATTCCTCCCGCGTCGCTATTCGAGCCAATAAGGTGGCGACACAAG   3120
GGAAAGATGCGCTGCAG                                               3137
```

Applicant's or agent's file reference number PC 375 I CTH	International application No

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 12 to 13, line 31 to 8

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depository institution

The National Collections of Industrial and Marine Bacteria Limited (NCIMB)

Address of depository institution (including postal code and country)

23 St Machar Drive
Aberdeen
AB2 1RY
United Kingdom

Date of deposit	Accession Number
7 March 1996	NCIMB 40789

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will only be made available either until the publication of the mention of the grant of the patent or after twenty years from the date of filing if the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer: Mrs. T. Brôcker-Tazelaar	Authorized officer

Form PCT/RO/134 (July 1992)

Applicant's or agent's file reference number	International application No
PC. 376 1 CTH	

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 33, line 12 - 21

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

The National Collections of Industrial and Marine Bacteria Limited (NCIMB)

Address of depositary institution *(including postal code and country)*

23 St Machar Drive
Aberdeen
AB2 1RY
United Kingdom

Date of deposit	Accession Number
7 March 1996	NCIMB 40790

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will only be made available either until the publication of the mention of the grant of the patent or after twenty years from the date of filing if the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer: Mrs. T. Bröcker-Tazelaar	Authorized officer

Form PCT/RO/134 (July 1992)

CLAIMS

1. An enzyme having α(1,4) glucan acetyl-transferase activity, wherein the enzyme comprises the amino acid sequence shown as SEQ ID No. 1, or a variant, homologue or fragment thereof.

2. A recombinant enzyme having α(1,4) glucan acetyl-transferase activity, wherein the enzyme comprises the amino acid sequence shown as SEQ ID No. 1, or a variant, homologue or fragment thereof.

3. A recombinant enzyme having α(1,4) glucan acetyl-transferase activity, wherein the enzyme has the amino acid sequence shown as SEQ ID No. 1.

4. A recombinant enzyme having α(1,4) glucan acetyl-transferase activity, wherein the recombinant enzyme is immunologically reactive with an antibody raised against a purified recombinant enzyme according to claim 3.

5. A nucleotide sequence coding for the enzyme of any one of claims 1 to 4 or a sequence that is complementary thereto.

6. A nucleotide sequence according to claim 5, wherein the nucleotide sequence is a DNA sequence.

7. A nucleotide sequence comprising the sequence shown as SEQ ID No. 2, or a variant, homologue or fragment thereof or a sequence that is complementary thereto.

8. A nucleotide sequence having the sequence shown as SEQ ID No. 2.

9. A construct comprising or expressing the invention according to any one of claims 1 to 8.

10. A vector comprising or expressing the invention of any one of claims 1 to 9.

11. A plasmid comprising or expressing the invention of any one of claims 1 to 10.

12. A transgenic organism comprising or expressing the invention according to any one of claims 1 to 11.

13. A transgenic organism according to claim 12, wherein the transgenic organism is a plant.

14. A modified carbohydrate (preferably starch) prepared by a method comprising or expressing or using the invention according to any one of claims 1 to 13.

15. An enzyme substantially as described herein.

FIGURE 1


```
                                        ATGAGCACAGAAAAAGAAAAGATGATTGCTGGT   1680
GAGTTGTATCGCTCGGCAGATGAGACGTTATCTCGCGATCGCCTGCGCGCTCGTCAGCTT   1740
ATTCACCGATACAATCATTCCCTGGCGGAAGAGCACACATTACGCCAGCAAATTCTCGCT   1800
GATCTATTCGGTCAGGTGACAGAGGCTTATATTGAGCCAACGTTTCGCTGTGACTATGGC   1860
TATAACATTTTTTCTCGGTAATAATTTTTTTCGCCAACTTCGATTGCGTGATGCTTGATGTC 1920
TGCCCTATTCGCATCGGTGATAACTGTATGTTGGCACCAGGCGTTCATATCTACACGGCA   1980
ACACATCCCATCGACCCTGTAGCACGTAATAGCGGTGCTGAACTGGGGAAACCCGTCACC   2040
ATCGGTAATAACGTCTGGATTGGCGGACGCGCGGTCATTAACCCTGGTGTGACCATTGGT   2100
GATAACGTCGTGGTAGCCTCAGGTGCAGTTGTCACAAAAGATGTCCCGGACAACGTTGTC   2160
GTGGGCGGTAATCCAGCCAGAATAATTAAAAAATTGTAA
```

FIGURE 2

```
MSTEKEKMIAGELYRSADETLSRDRLRARQLIHRYNHSLAEEHTLRQQIL   50

ADLFGQVTEAYIEPTFRCDYGYNIFLGNNFFANFDCVMLDVCPIRIGDNC   100

MLAPGVHIYTATHPIDPVARNSGAELGKPVTIGNNVWIGGRAVINPGVTI   150

GDNVVVASGAVVTKDVPDNVVVGGNPARIIKKL                     183
```

FIGURE 3


```
                                                                    60
GAATTCGCCAAAGACTTGATGGATAAAGAAGGTAAAGGTCTGATTGAAGCGACGCTTGAT
GCGGTGCGGATGCGTTTACGTCCGATCCTGATGACCTCGCTGGCGTTTATCCTCGGCGTT      120
ATGCCGCTGGTTATCAGTACTGGTGCTGGTTCCGGCGCGCAGAACGCAGTAGGTACCGGT      180
GTAATGGGCGGGATGGTGACCGCAACGGTACTGGCAATCTTCTTCGTTCCGGTATTCTTT      240
GTGGTGGTTCGCCGCCGCTTTAGCCGCAAGAATGAAGATATCGAGCACAGCCATACTGTC      300
GATCATCATTGATACAACGTGTAATCACTAAGGCCGCGTAAGCGGCCTTTTTTATGCATA      360
ACCTACGAACATTAAGGAGTAATTGAACCACCAACTCAGGATCTCATACGAAAACCAGTA      420
TTAACCACGGATAAAATTCATAAAAAATACTGATTGTTAGTTAATTTATATTAAGTAGCG      480
CTAATAGATTTAATAATCCATAATCATTTAGAGGCTATTCTTAATTATTTGCGGTAATTC      540
TTTATTCATTCCTCGGTTATTACGTCATATTCAGAGCAATCCTGGTATTAGTGTCACCAA      600
TTTCATCTGGCGATAATCCTGAAATGTTATGAATAGTTCGAGCAAACTGCTTTTACCTGC      660
TGCGGGTTAGTGCTAGTATGAAAAAGTGAGTCCTGTCCCGCTTCCTTCCTAATTGTAATT      720
TTTCGTAATAATGCGATGAAAACCTGCAAAGAGTGGCTTATAGTTAAGCTAACAAACGAG      780
AGGGCAAGTCCAGGTCAGTAAGTTTTTTCCATCCCGAAAGGTGTCCGTTAGTTCAACCGC      840
TAAGAAGGGGACGCGTTATGGATGAATACTCACCCAAAAGACATGATATCGCACAGCTTA      900
AGTTTCTCTGTGAAACCCTGTATCATGACTGCCTTGCAAACCTTGAAGAAAGCAATCATG      960
GCTGGGTAAACGACCCAACCTCGGCGATCAACCTCCAGTTGAATGAACTGATTGAGCATA     1020
TTGCGACCTTCGCACTTAATTACAAAATTAAGTATAATGAAGACAATAAGCTCATTGAGC     1080
AGATCGACGAATATCTGGATGACACCTTTATGTTGTTCAGTAGTTATGGTATTAATATGC     1140
AGGATCTTCAGAAATGGCGGAAGTCAGGTAAHCGACTATHCCGTTGTTTTGTCAATGCGA     1200
CGAAAGAGAATCCTGCGAGTTTATCTTGTTAGAATTATTACAACCATAGGTAGAAGTATG     1260
TCCGAAAAACCTTTAACGAAAACCGATTATTTAATGCGTTTACGTCGTTGCCAGACAATT     1320
GACACGCTGGAGCGGTTTAWTCGAGAAAAATAAATACGAATTATCAGATAATGAACTGGC     1380
GGTATTTTACTCAGCCGCAGATCACCGCCTCGCCGAATTGACCATGAATAAACTGTACGA     1440
CAAGATCCCTTCCTCAGTATGGAAATTTATTCGCTAATAAATAATTCGCTTTCGGAGCTA     1500
TAACCGGCTGTTTATTAAGAATTTTATACTTTTTCGCCATGAAGACATACCCTATGTGAT     1560
CTTTATCACACAGATGTAATGGGAACGTTCTCTTCACTGACTTTTCGTCTTACTGTGTTG     1620
CCGCATTTTCAGCAACCGGAGTCAGTAATGAGCACAGAAAAAGAAAAGATGATTGCTGGT     1680
                                M  S  T  E  K  E  K  M  I  A  G
GAGTTGTATCGCTCGGCAGATGAGACGTTATCTCGCGATCGCCTGCGCGCTCGTCAGCTT     1740
E  L  Y  R  S  A  D  E  T  L  S  R  D  R  L  R  A  R  Q  L
ATTCACCGATACAATCATTCCCTGGCGGAAGAGCACACATTACGCCAGCAAATTCTCGCT     1800
I  H  R  Y  N  H  S  L  A  E  E  H  T  L  R  Q  Q  I  L  A
GATCTATTCGGTCAGGTGACAGAGGCTTATATTGAGCCAACGTTTCGCTGTGACTATGGC     1860
D  L  F  G  Q  V  T  E  A  Y  I  E  P  T  F  R  C  D  Y  G
TATAACATTTTTCTCGGTAATAATTTTTTTCGCCAACTTCGATTGCGTGATGCTTGATGTC     1920
Y  N  I  F  L  G  N  N  F  F  A  N  F  D  C  V  M  L  D  V
TGCCCTATTCGCATCGGTGATAACTGTATGTTGGCACCAGGCGTTCATATCTACACGGCA     1980
C  P  I  R  I  G  D  N  C  M  L  A  P  G  V  H  I  Y  T  A
ACACATCCCATCGACCCTGTAGCACGTAATAGCGGTGCTGAACTGGGGAAACCCGTCACC     2040
T  H  P  I  D  P  V  A  R  N  S  G  A  E  L  G  K  P  V  T
ATCGGTAATAACGTCTGGATTGGCGGACGCGCGGTCATTAACCCTGGTGTGACCATTGGT     2100
I  G  N  N  V  W  I  G  G  R  A  V  I  N  P  G  V  T  I  G
```

FIGURE 3 Continued


```
GATAACGTCGTGGTAGCCTCAGGTGCAGTTGTCACAAAAGATGTCCCGGACAACGTTGTC   2160
D  N  V  V  V  A  S  G  A  V  V  T  K  D  V  P  D  N  V  V
GTGGGCGGTAATCCAGCCAGAATAATTAAAAAATTGTAATCGGTTTTTCGCAACTGTTAT   2220
V  G  G  N  P  A  R  I  I  K  K  L
GCAAAATTGTGGTAGATCTGTTACTTCCCCTCTACTATTCCCACGTTAAAATAGGGTGTT   2280
CCCTGGAAAGTTGCAGATACCACGAAGGCAAACGATGACCGAAATACAACGCCTGCTGAC   2340
CGAAACGATTGAGTCTCTGAATACCCGCGAAAAACGCGACAACAAACCCCGCTTTAGTAT   2400
CAGTTTTATCCGTAAACATCCGGGGCTGTTTATCGGTATGTACGTTGCTTTTTTTGCCAC   2460
CCTGGCGGTGATGTTGCAGTCCGAAACGCTGTCAGGCTCTGTCTGGCTACTGGTTGTATT   2520
ATTTATCCTGCTTAATGGTTTCTTCTTTTTCGATGTCTACCCACGCTACCGCTATGAAGA   2580
TATCGACGTGCTGGATTTCCGCGTTTGCTATAACGGCGAATGGTACAACACGCGCTTTGT   2640
ACCTGCCGCGCTGGTTGAAGCCATCTTGAACTCTCCGTGTCGCGGATGTTCATAAGGAAC   2700
AACTGCAAAAAATGATCGTCCGTAAAGGTGAACTGTCTTTTTACGATATTTTTACCCTCS   2760
TCGCGCCGAATCAACATCTTAAGTTAGGGTTACATACCAGGCGTAAAGCTCTGCGCCTGG   2820
TCAAATGACAATGATCGTTTCCACCCATCACTTCATGAAATACCAGCTCTACCTCCTTAT   2880
CTCCAGCCAGCCTTTTTCCACAATCAGATATACTTTCCCTACACTGTGTTAATAAGGATA   2940
TGCTGGTGAGAACACGACATCTGGTCGGCCTTATTTCGGGAGTACTGATTCTTTCAGTAT   3000
TGCTGCCTGTCGGCTTAAGCATCTGGCTGGCCCATCAGCAGGTAGAAACATCGTTTATTG   3060
AAGAGCTGGATACCTATTCCTCCCGCGTCGCTATTCGAGCCAATAAGGTGGCGACACAAG   3120
GGAAAGATGCGCTGCAG                                               3137
```

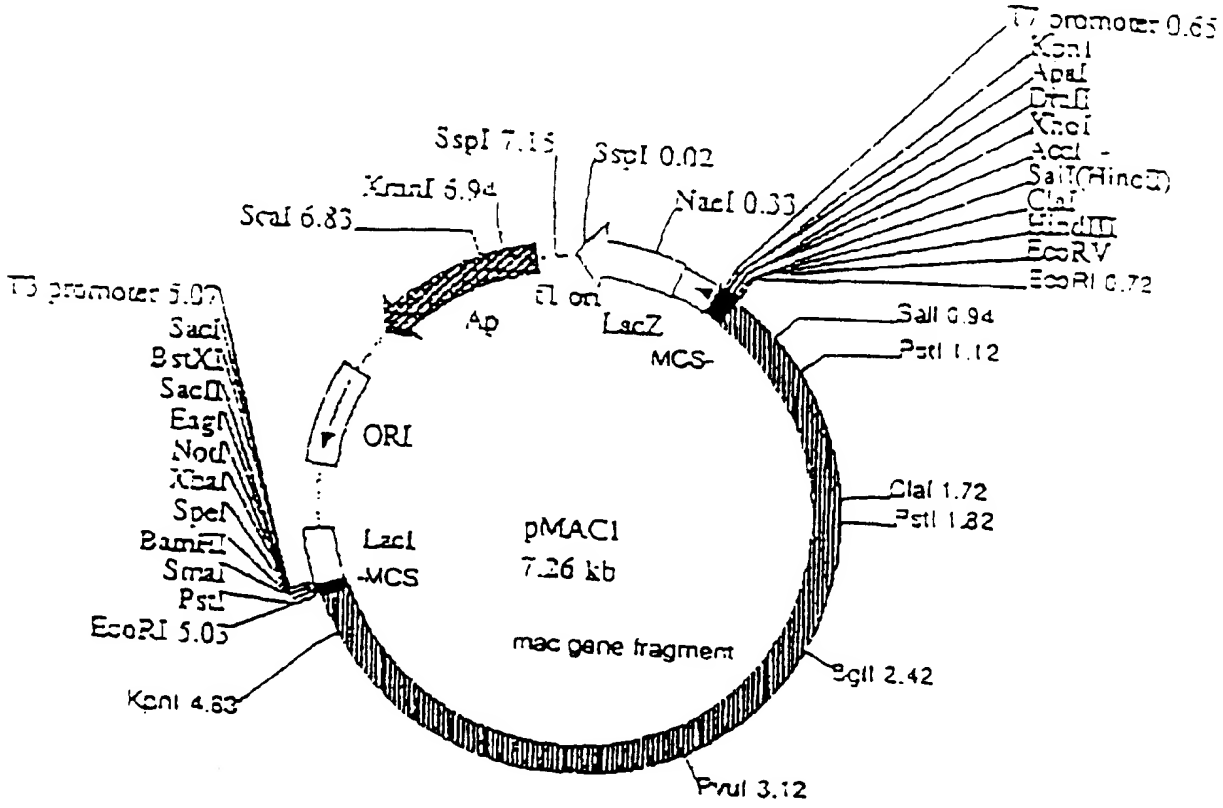
T7 promoter 0.65
KonI
ApaI
DraII
XhoI
AccI
SalI(HincII)
ClaI
HindIII
EcoRV
EcoRI 0.72
SspI 7.15
SspI 0.02
NaeI 0.33
KpnI 6.94
ScaI 6.83
f1 on
Ap
LacZ
MCS
SalI 0.94
PstI 1.12
T3 promoter 5.07
SacI
BstXI
SacII
EagI
NotI
XbaI
SpeI
BamHI
SmaI
PstI
EcoRI 5.05
KonI 4.83
ORI
LacI
MCS
pMAC1
7.26 kb
mac gene fragment
ClaI 1.72
PstI 1.82
BglI 2.42
PvuI 3.12

Fig 4

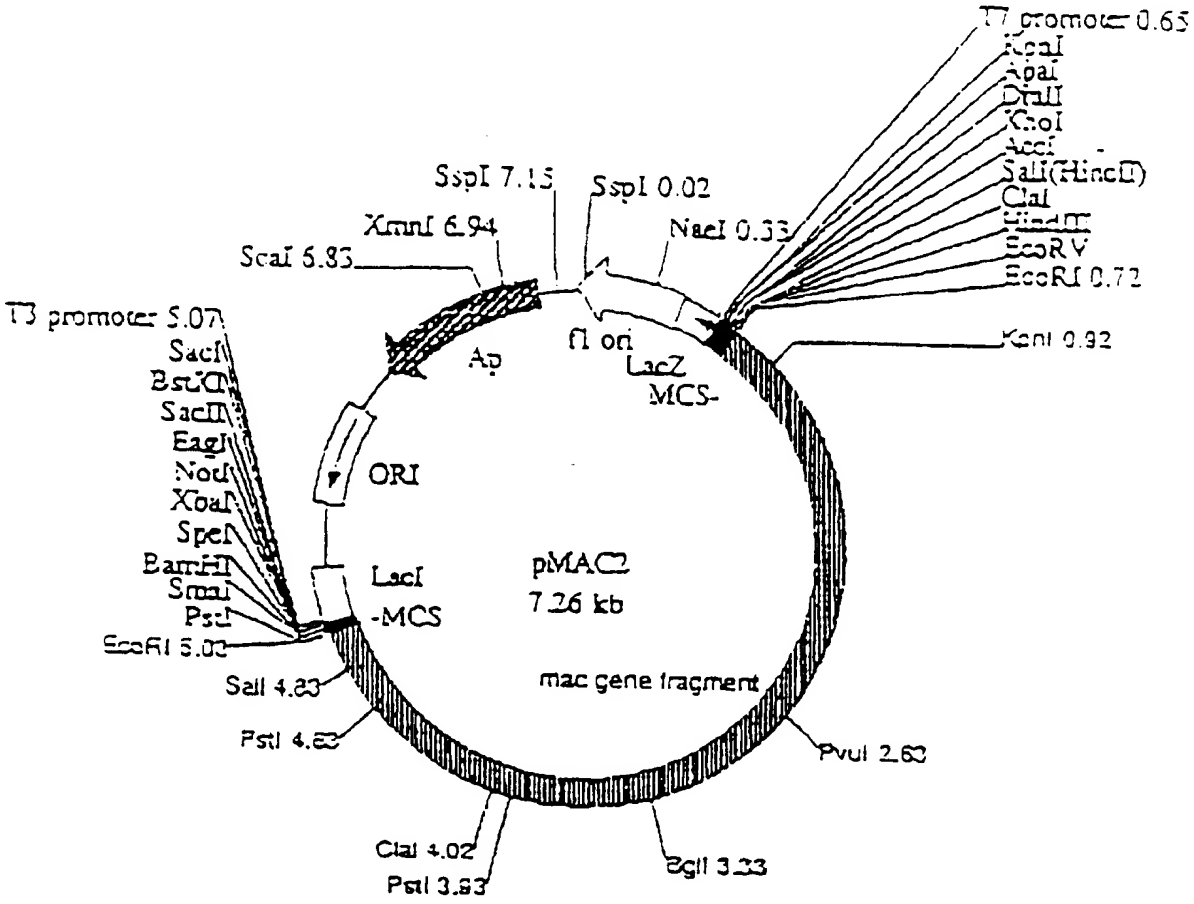
T7 promoter 0.65
XcmI
ApaI
DraII
XhoI
AccI
SalI(HincII)
ClaI
HindIII
EcoRV
EcoRI 0.72
KpnI 0.92
SspI 7.15
SspI 0.02
NaeI 0.33
XmnI 6.94
ScaI 5.83
T3 promoter 5.07
SacI
BstXI
SacII
EagI
NotI
XbaI
SpeI
BamHI
SmaI
PstI
EcoRI 5.02
SalI 4.83
PstI 4.63
ClaI 4.02
PstI 3.83
BglI 3.33
PvuI 2.62
Ap
f1 ori
LacZ
MCS-
ORI
LacI
-MCS
pMAC2
7.26 kb
mac gene fragment

Fig 5

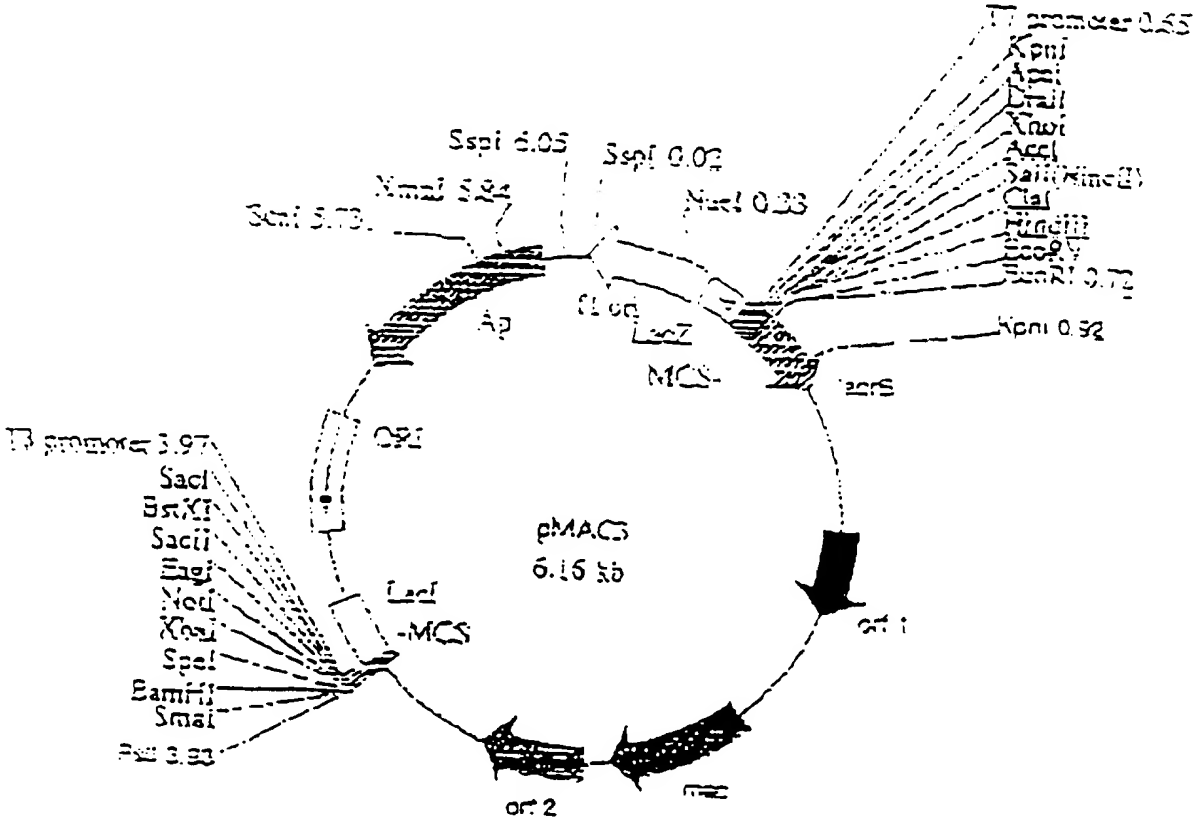
T7 promoter 0.65
KpnI
ApaI
BstEII
XhoI
AccI
SalI
ClaI
HindIII
EcoRV
SmaI 0.72
KpnI 0.92
SspI 6.05
SspI 0.02
NaeI 0.33
XmaI 5.84
SacI 5.78
Ap
f1 ori
LacZ
MCS
lacrS
ori
T3 promoter 3.97
SacI
BstXI
SacII
EagI
NotI
XhoI
SpeI
BamHI
SmaI
PstI 3.83
LacI
-MCS
pMACS
6.16 kb
ori 1
ori 2

Fig 6

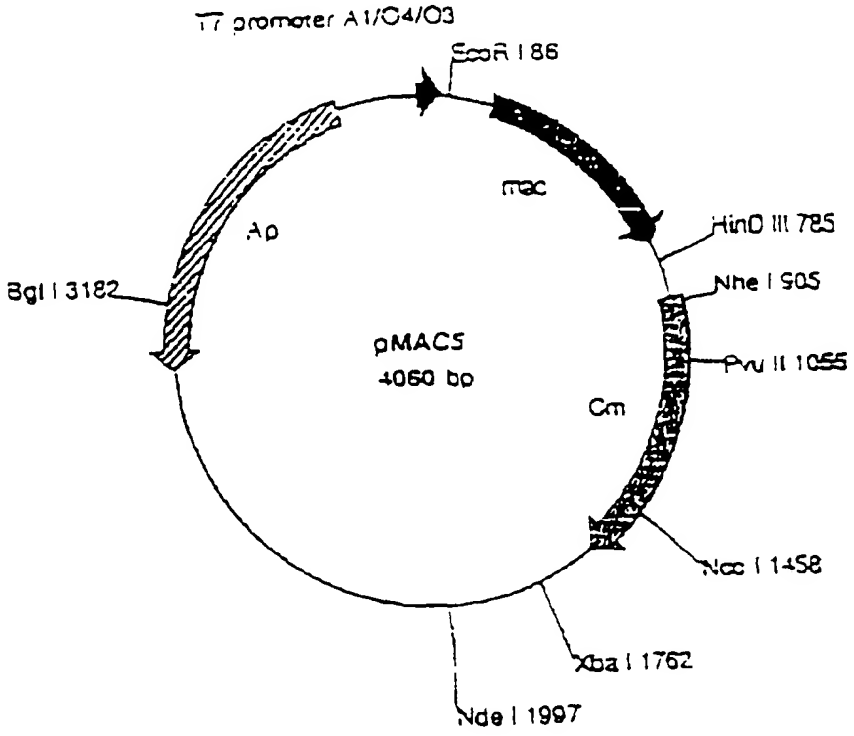
T7 promoter A1/O4/O3
EcoR I 86
mac
HinD III 785
Nhe I 905
Pvu II 1055
Cm
Ncc I 1458
Xba I 1762
Nde I 1997
Ap
Bgl I 3182
pMACS
4060 bp

Fig 7

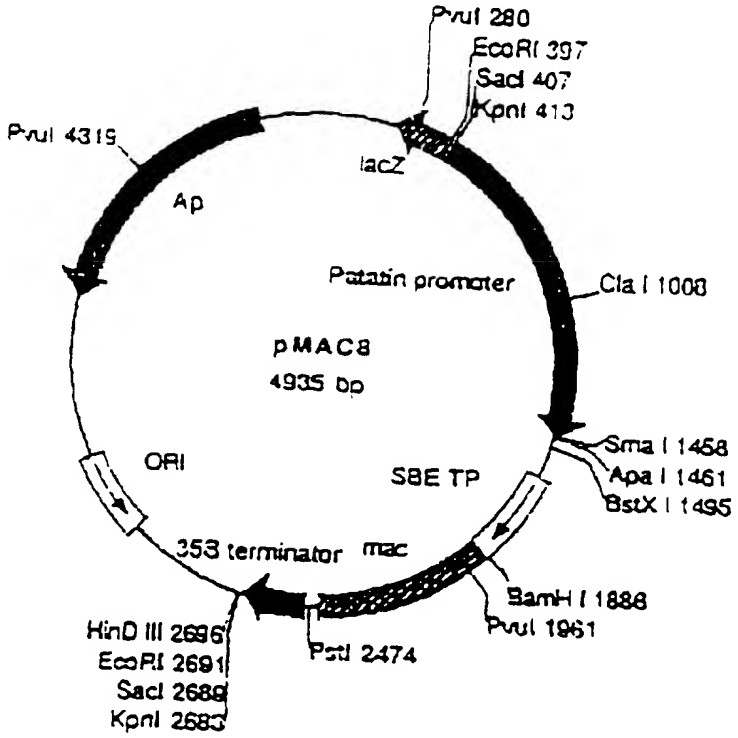
PvuI 280
EcoRI 397
SacI 407
KpnI 413
PvuI 4319
lacZ
Ap
Potatin promoter
ClaI 1008
pMAC8
4935 bp
SmaI 1458
ApaI 1461
BstXI 1495
ORI
SBE TP
35S terminator
mac
BamHI 1888
PvuI 1961
HinDIII 2696
EcoRI 2691
SacI 2689
KpnI 2683
PstI 2474

Fig 8

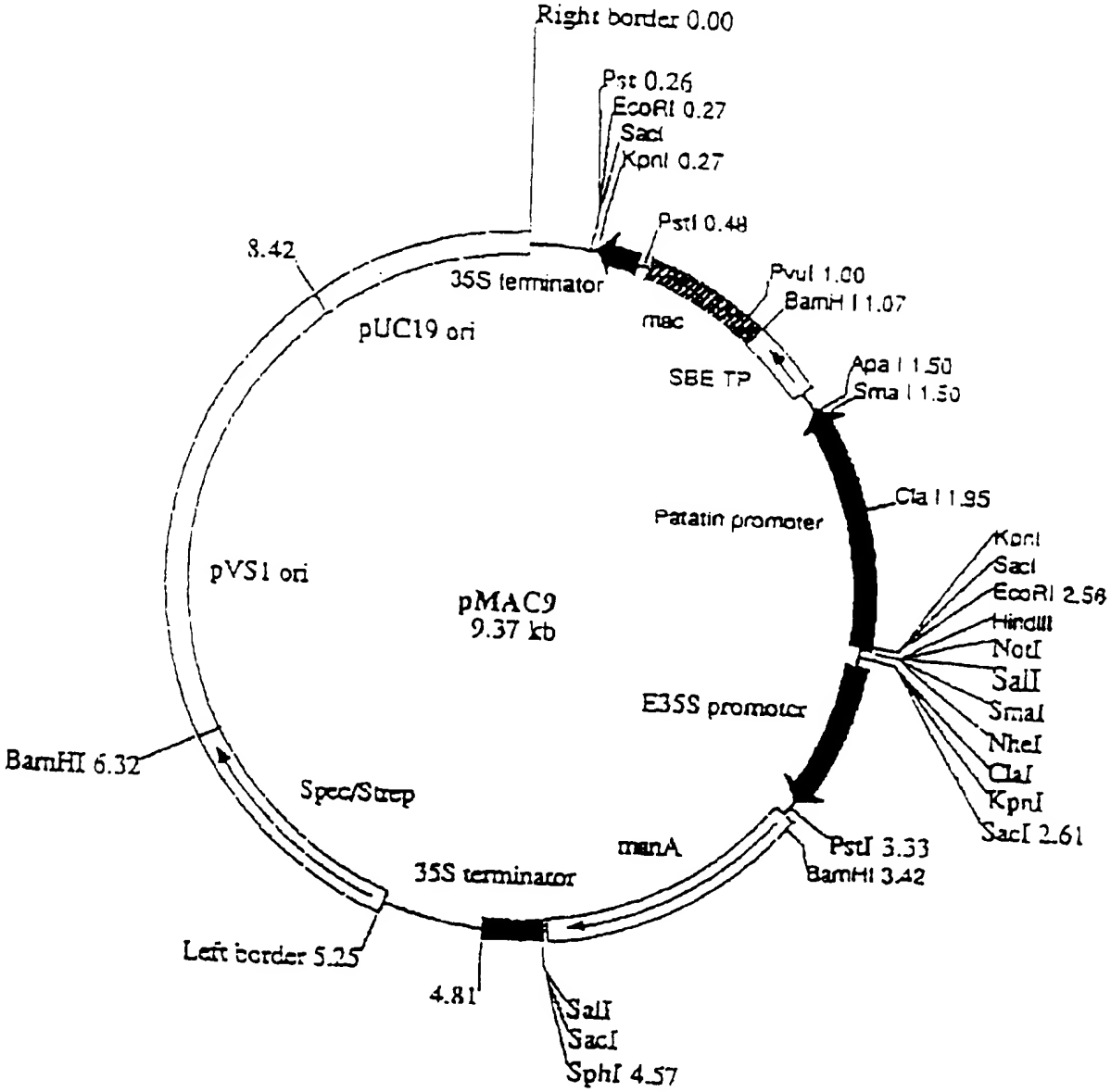
Right border 0.00
Pst 0.26
EcoRI 0.27
SacI
KpnI 0.27
PstI 0.48
8.42
35S terminator
pUC19 ori
mac
PvuI 1.00
BamH I 1.07
SBE TP
ApaI 1.50
SmaI 1.50
ClaI 1.95
Patatin promoter
KpnI
SacI
EcoRI 2.56
HindIII
NotI
SalI
SmaI
NheI
ClaI
KpnI
SacI 2.61
pVS1 ori
pMAC9
9.37 kb
E35S promoter
BamHI 6.32
Spec/Strep
manA
PstI 3.33
BamHI 3.42
35S terminator
Left border 5.25
4.81
SalI
SacI
SphI 4.57

Fig 9

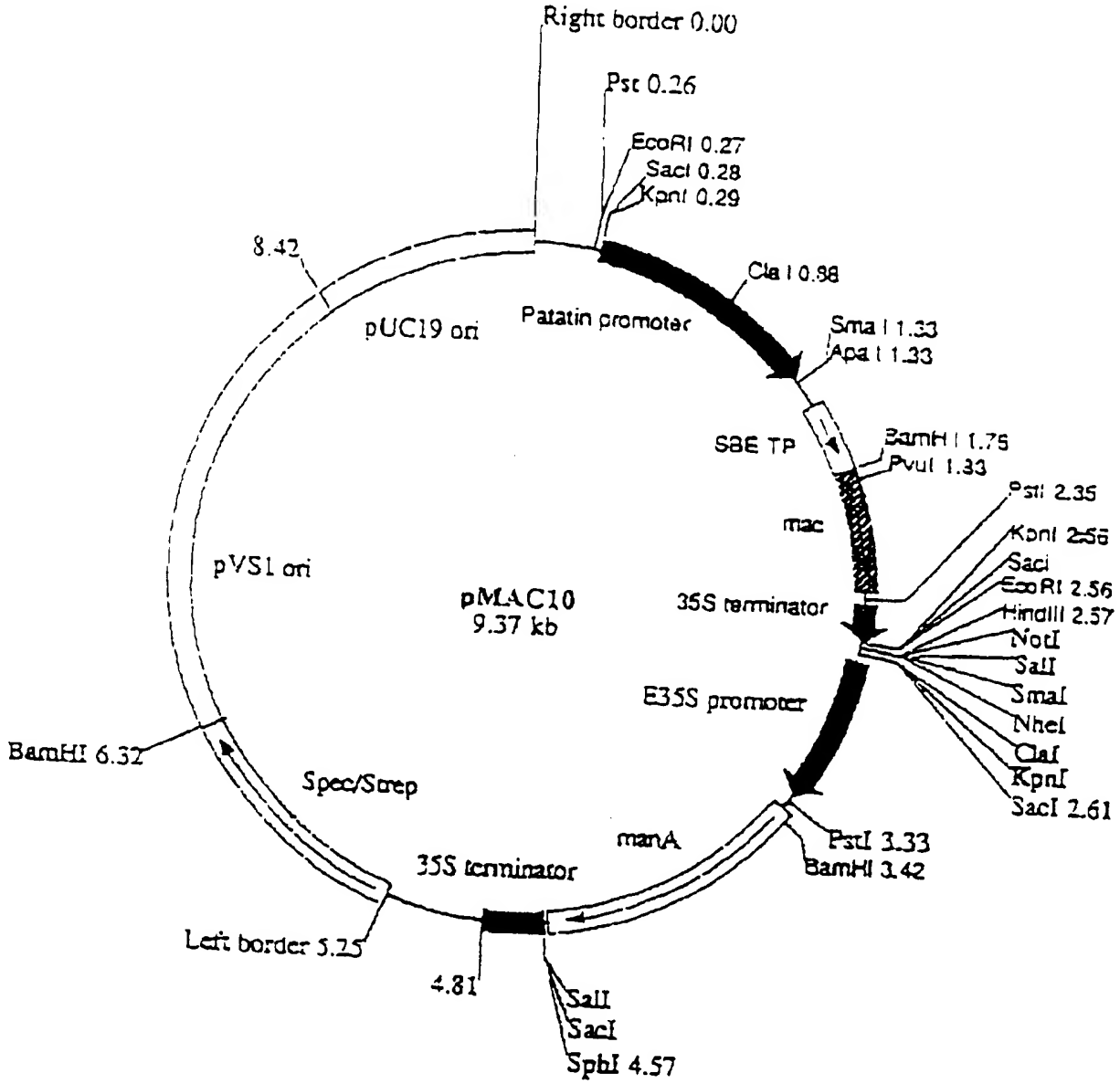
Right border 0.00
Pst 0.26
EcoRI 0.27
SacI 0.28
KpnI 0.29
8.42
ClaI 0.98
pUC19 ori
Patatin promoter
SmaI 1.33
ApaI 1.33
SBE TP
BamHI 1.75
PvuI 1.33
PstI 2.35
mac
KpnI 2.56
SacI
EcoRI 2.56
HindIII 2.57
NotI
SalI
SmaI
NheI
ClaI
KpnI
SacI 2.61
pVS1 ori
pMAC10
9.37 kb
35S terminator
E35S promoter
BamHI 6.32
Spec/Strep
manA
PstI 3.33
BamHI 3.42
35S terminator
Left border 5.25
4.81
SalI
SacI
SphI 4.57

Fig 10